Acta Biomaterialia 8 (2012) 4208–4214

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Transfection of macrophages by collagen hollow spheres loaded with polyplexes: A step towards modulating inflammation ☆

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ARTICLE INFO

Article history:
Available online 15 June 2012

Keywords:
Chronic inflammation
Macrophages
Collagen microspheres
Polyplexes
Gene delivery

ABSTRACT

Macrophages are key orchestrators of inflammation as they secrete proteases and inflammatory cytokines. To date, therapies aimed at modulating macrophage phenotype have failed due to the short half-life of biomolecules in the body. Therefore, inhibition of inflammation by gene therapy constitutes a new hope.

In the present study, we have assessed collagen hollow spheres as a reservoir system for polyplexes in order to transfect human macrophages while preserving cell viability. Polyplexes were formed by complexing G-Luc plasmid with a poly(2-dimethylaminoethyl methacrylate) poly(ethylene glycol) based hyperbranched polymer. Several ratios of polymer/pDNA (5:1, 8:1, 10:1 w/w) complexes in two different sphere sizes (1.24 and 4.5 μm) were tested. Collagen hollow spheres were loaded with polyplexes up to 80 μg of pDNA per mg of microspheres. The release of polyplexes from the spheres was delayed and prolonged i.e. 20% of the initial amount released in 5 days. Following incubation with polyplex-loaded microspheres, macrophages were transfected (polyplex pDNA:polymer ratio 1:10 w/w). In addition, collagen hollow spheres maintained cell viability as more than 80% of cells were viable after 4 days in culture. In contrast, when used alone, polyplexes were seen to be toxic, while there was no transfection detected. Taken together, these results show that collagen hollow spheres may be used as a reservoir for controlled gene delivery to macrophages. Unlike existing gene delivery systems, this system allows for macrophage transfection with minimal toxicity. Hence, this system has a potential for the delivery of a therapeutic gene in order to modulate inflammation.

1. Introduction

Macrophages are key cells in the resolution of inflammation. During the inflammatory phase following injury, macrophages adopt a classically activated phenotype characterized by the secretion of reactive oxygen species, inflammatory cytokines and proteases [1,2]. Following exposure to biological signals such as interleukin (IL)-13, IL-4 or IL-10, macrophages progressively change their phenotype to adopt an alternatively activated phenotype. This phenotype promotes wound healing by the secretion of IL-10, VEGF and TGF-β1 to suppress the inflammatory response and promote matrix formation and stabilization [2]. In the case of many chronic inflammatory conditions, macrophages are locked in a pro-inflammatory phenotype [3].

With the aim of modulating inflammation, several trials based on the injection of growth factors have been performed. Unfortunately, owing to the short half-life and the rapid diffusion of biomolecules in vivo [4,5], these attempts have thus far proved unsuccessful. Hence, gene therapy in the form of the transfection of a gene encoding for a protein capable of modulating inflammation presents a new possibility to modulate the pro-inflammatory environment. Unfortunately macrophages, which are a non-dividing cell type, are difficult to transfect. Firstly, viral transfecting reagents cannot be used as they trigger an immune response [6]. Secondly, pDNA barely penetrates into the nucleus of non-dividing cells. Lastly, regular non-viral reagents reduce macrophage viability after transfection [7,8].

In gene therapy research, the utilization of cationic polymers has proven to have several advantages when compared to cationic lipids. Cationic polymers are more stable than lipids and complex a large amount of pDNA to form positively charged polyplexes. Unfortunately, however, they are toxic for macrophages [8]. To overcome this, polymeric hollow spheres made from natural polymers have been developed as a gene depot system [9]. These systems allow for the sustained release of polyplexes, which can

☆ Part of the Special Issue "Advanced Functional Polymers in Medicine (AFPM)", guest editors: Professors Luigi Ambrosio, Dirk W. Grijpma and Andreas Lendlein.

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http://dx.doi.org/10.1016/j.actbio.2012.06.017

protect macrophages from the toxicity of large, bolus doses of polyplexes. Several studies have shown that hollow spheres can be fabricated from natural polymers using a template method with sizes on the nanometer to micrometer scale possible [10]. Hollow spheres made from natural polymers have the advantage that they will not release toxic degradation products and are not detrimental to cell viability [11]. This technique relies on the coating of polystyrene beads by natural polymers such as chitosan or elastin-like peptides due to an electrostatic interaction between the negatively charged polystyrene beads and the positively charged polymers in solution. After the removal of the polystyrene bead template using the solvent tetrahydrofuran (THF), hollow spheres can be obtained. These studies demonstrate that spheres can be efficiently loaded with polyplexes, which modulates the polyplex release pattern and allows for transfection of cells in vitro [11]. In addition, sustained release of polyplexes protected cells from polymer cytotoxicity. Recently, Browne and collaborators fabricated a new type of hollow sphere made from atelocollagen type I [13]. This type of sphere is easy to fabricate and can be loaded with a large amount of polyplexes. It was hypothesized in this study that a reservoir system composed of atellocollagen type 1 can load and release polyplexes in a consistent and controlled manner, with the released polyplexes capable of transfection while protecting macrophages against polymer toxicity associated with large doses. The objective of this study was to use collagen hollow spheres as a reservoir for polyplexes to transfect human macrophages.

2. Materials and methods

2.1. Collagen hollow sphere fabrication

Collagen hollow spheres of defined sizes were fabricated using the template method as previously described [13]. Briefly, 1.2 or 4.5 μm sulfonated polystyrene beads were incubated with an atelocollagen® solution (5 mg ml^{-1} in 0.5 M acetic acid) for 4 h (supplementary information no. 1). After cross-linking with pentaerythritol poly(ethylene glycol) ether tetrasuccinimidyl glutarate 4 arm-StarPEG for 2 h, the polystyrene core was removed with THF, leaving an atellocollagen hollow sphere of defined size.

2.2. Characterization by scanning electron microscopy (SEM)

Prior to analysis, collagen hollow spheres were fixed with 4% paraformaldehyde. A drop of sample containing collagen hollow spheres was placed on adhesive carbon tabs mounted on SEM specimen stubs and then dried. The specimens were subsequently coated with gold using an Emitech K550 coating system. SEM images were obtained using a Hitachi S-4700 field emission microscope operating with a beam voltage of 15 kV.

2.3. Polyplex formation

Polyplexes were formed by complexing the synthesized polymer (B81) with G-Luc plasmid encoding for the protein Gaussia Luciferase (New England Biolabs, Ireland). The transfecting agent B81 (14 kDa) synthesized in our group is composed of a linear poly(2-dimethylaminoethyl methacrylate) block (pDMAEMA), a hyperbranched poly(ethylene glycol methyl ether methacrylate) block and an ethylene dimethacrylate block (see structure in supplementary information no. 2). The polymer B81 was synthesized via the deactivation enhanced atom transfer radical polymerization technique. Polyplexes were formed in phosphate-buffered saline (PBS) with weight ratios from 5:1 to 10:1 (Polymer B81/G-Luc plasmid) for 1 h. To assess the transfection efficiency with the polymer B81, we compared the results with those of the commercial polymers: polyethylenimine 25 kDa (PEI; Sigma, Ireland) and Superfect® (Qiagen, Ireland). Linear PEI 25 kDa was used with an optimal ratio of 2:1 (polymer/G-Luc plasmid) and the partially degraded polyamido amine dendrimer (Superfect®) was used with an optimal ratio of 8:1.

2.4. Analysis of polyplexes

The size, zeta potential and polydispersity index of polyplexes were measured by Zetasizer (Malvern Instruments Zetasizer Nano-2590). This analysis was performed on polyplexes formed with the polymer B81 after 12 h of incubation.

2.5. Loading studies

One mg of 1.2 or 4.5 μm collagen hollow spheres was resuspended in 300 μl of PBS. Polyplexes (ratio 10:1 w/w) containing a predetermined amount of pDNA (40, 80, 160 and 320 μg) were added to these spheres, followed by a volume of THF to achieve a final concentration of 50% (v/v). The mixtures were then agitated for 6 h at room temperature. After this period of incubation, the vials were opened to let the THF evaporate for 6 h. Next, the suspensions were centrifuged at 13,000*g* and the sphere/polyplex complexes were washed four times with ultrapure water. The supernatants collected were used to estimate the amount of free pDNA. A PicoGreen® assay (Invitrogen, Ireland) was performed to quantify free pDNA. To obtain free pDNA, polyplex samples collected during loading were treated with polyglutamic acid (PGA) of 10 mg ml^{-1} concentration at 37 °C for 30 min with a modification of the protocol described in Ref. [11]. Then 100 μl of PicoGreen® was added to each sample of similar volume and the fluorescence was analyzed at 480 nm. A standard curve was prepared using naked pDNA. First the amount of pDNA detected in the supernatants was determined, then the loading efficiency of the hollow spheres was determined using the formula:

$$\text{Loading efficiency} = \frac{\text{Initial pDNA added} - \text{pDNA remaining in supernatant}}{\text{Initial pDNA added}}$$

2.6. Release pattern of polyplexes from collagen hollow spheres

To characterize the release pattern of the polyplex-loaded collagen hollow spheres, 1 mg of polyplex-loaded spheres of 1.2 or 4.5 μm size was resuspended in PBS and incubated at 37 °C. The spheres used for this experiment were loaded with 40 μg of pDNA per mg of spheres. At each time point, the suspension was centrifuged and a sample of the supernatant taken. The released polyplexes were treated with polyglutamic acid (Sigma, Ireland) and quantified using the PicoGreen® (Invitrogen, Ireland) assay. To determine the release pattern, five time points were considered: days 1, 2, 3, 4 and 5. The results were expressed as a percentage of initial amounts of pDNA loaded in the spheres.

2.7. Macrophage differentiation and activation

The human myelogenous leukemia cell line THP-1 was obtained from the ATCC. The cells were maintained in RPMI 1640 (GIBCO-BRL) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37 °C in a 5% CO_2 humidified incubator. The mature macrophage-like state was induced by treating THP-1 cells for 24 h with phorbol 12-myristate 13 acetate (PMA) at 100 ng ml^{-1} diluted in serum-free medium. Cells were seeded at 100,000 cells well^{-1} into a 48-well plastic well plate. The following day, plastic-adherent cells were washed twice with cold, sterile Dulbecco's PBS and incubated with fresh